의학석사 학위논문

Monitoring of Porcine Endogenous Retrovirus (PERV) in the Xenotransplanted Primates

영장류 이종이식에서 돼지 내인성 레트로바이러스의 모니터링

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사 모 아

A thesis of the Master's degree

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Department of Microbiology and Immunology,

Seoul National University College of Medicine

Graduate School

Moa Sa

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지도 교수 황 응 수

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Monitoring of Porcine Endogenous Retrovirus (PERV) in the Xenotransplanted Primates

by Moa Sa

(Supervised by Prof. Eung-Soo Hwang)

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in partial fulfillment of the requirement
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Approved by Thesis Committees:

Professor Chul Chung Kim **Chairman**

Professor Eung-Soo Hwang **Vice chairman**

Professor Bu-Joon Kim

ABSTRACT

The infection risk of porcine endogenous retrovirus (PERV) to human is considered an obstacle in the field of pig-to-human xenotransplantation. Therefore, pre-screening and post-monitoring of PERV in donor pigs and transplanted recipients respectively are critical in the aspect of biosafety. The development of multiplex real-time PCR technology was applied for the detection of PERV *pol* to determine the PERV infectivity, pig mitochondrial cytochrome oxidase II (COII) to distinguish the contamination of pig cells, and monkey glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to use as an internal control. Although the primers and probes were chosen based on the consensus sequences from SNU miniature pigs, the performances of quantitative multiplex PCR assays were effective in the cells from other pig strains. In addition, the detection limits of the singleplex and multiplex methods were 5-10 copies per 20μl reaction which was similar to the detection limits of nested PCR. Even though the sensitivity was slightly lower in multiplex real-time PCR, it had the advantage of reactions in one tube to reduce error rate in case of the usage in different tubes. Multiplex real-time PCR assays performed on various pig breeds determine the ratio of PERV *pol* to pig COII. The average of *pol* ratio in three SNU miniature pigs was 5.8-fold higher than the ratio in PK15 cell line. Also, the amplification on

xenotransplanted samples showed that PERV did not transmit to *Rhesus macaque* recipients. These results demonstrate that the triple labeled real-time PCR assay developed in this study can use as both a screening tool to examine PERV proviral load in donor pigs and a monitoring tool to examine PERV transmission in non-human primates (NHP) and human recipients after xenotransplantation.

Keywords: Xenotransplantation, Porcine endogenous retrovirus, Multiplex real-time PCR, Proviral load, Infectivity test

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LIST OF ABBREVIATIONS

α1, 3-GT: α1, 3-galactosyltransferase

BHQ: black hole quencher

COⅡ：mitochondrial cytochrome oxidase Ⅱ

C_T: threshold cycle

CV: coefficient of variation

Cy5: cyanine 5

DPF: designated pathogen-free

FAM: fluorescein

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

hCRPs: human complement regulatory proteins

HEK: human embryonal kidney

JOE: 4-5-dichloro carboxyfluorescein

NHP: non-human primates

NOD/SCID: non-obese diabetic, severe combined immunodeficiency

PBL: peripheral blood leukocytes

SD: standard deviation

SPF: specific pathogen-free

UNG: uracil deoxyribonucleic acid glycosylase

INTRODUCTION

The shortage of human donor organs in transplantation has turned away to animal donor for use in human recipients (1). The pig organs are regarded as the most promising source in the aspects of zoonotic infection, compared to non-human primates (NHP), and similarities to human in anatomy and physiology (2-4). Also, hyperacute rejection (HAR) has been overcome using α1, 3-Galactosyltransferase (α1, 3-GT)-knockout cloned pigs (5), and pigs expressing human complement regulatory proteins (hCRPs) (6). Since then, xenotransplantation of islets or solid organs has been tried on primates for pre-clinical trials (7-10). However, all pigs including specific pathogen-free (SPF) and designated pathogen-free (DPF) breeds impose porcine endogenous retrovirus (PERV) which is integrated in the pig genome (11, 12).

PERV is a simple gammaretrovirus composed of *gag, pol,* and *env* and classified into PERV-A, PERV-B and PERV-C according to *env* sequences (13). PERV-A and PERV-B have various host ranges including human (11, 14) but PERV-C infects only pig cells (13). Human-tropic PERV can infect human embryonal kidney (HEK) 293 cells, HeLa cells (15), and primary human endothelial cells productively *in vitro* (16). In addition, recombinant between PERV-A and PERV-C can be made and possess the tropism to human cells (17). Therefore, breeds with PERV-C are not recommended as

xenograft donors.

An *in vivo* test using non-obese, diabetic, and severe combined immunodeficiency (NOD/SCID) mice was reported that several tissues of them were infected by PERV and virus were expressed continuously (18). Most of the recipients were under immune suppressed condition, so PERV could be related with opportunistic pathogen like other retroviruses (19). As other retroviruses can integrate into a host genome, PERV can integrate into human gene (20).

Nevertheless, there was no evidence that PERV has infected primates or humans *in vivo* (21-23). Co-culture of pig cells with human cell lines also showed that PERV failed to replicate productively (24, 25). Follow-up in xenograft recipients did not exhibit the appearance of PERV infection after 52 weeks (26). In addition, the monitoring of 18 patients who had been transplanted with pig islets for nine years did not show any PERV infection (27). To date, these data mean that PERV has abilities to infect human cells and integrate it into other host genomes although productive replication of PERV in human *in vivo* has not been detected with it.

Chimera can be formed after transplantation when the recipient accepts the organ of lymphoid cells from other hosts and allows immune tolerance (28-30). The co-existence of xenograft and host cells makes the positive detection of PERV provirus on DNA level. The

microchimerism is defined when a small component of cell, such as mitochondria, migrates into other individual cells in the transplantation (31, 32). As such, the condition of PERV infection should be verified with the inclusion of one of the pig mitochondrial genes (24, 26).

According to the International Xenotransplantation Association (IXA) consensus statements, PERV should be screened for donor pigs and monitored for pre-clinical recipients (33). Several techniques can be used to detect the existence of PERV. Conventional PCR analyses and reverse transcriptase assays have been employed to measure the DNA and RNA level, respectively. To increase sensitivity and specificity, nested PCR (24, 34) and quantitative real-time PCR (35-37) targeting porcine housekeeping genes and PERV conserved sequences have been performed to detect a low-copy number of PERV.

The objectives of this study were to monitor the PERV proviral level and transmission of PERV in NHP xenotransplanted with pig islets or corneas with the multiplex real-time PCR for the detection of PERV *pol*, COⅡ and monkey GAPDH.

MATERIALS AND METHODS

1. Sample Preparation

1-1. Cell Lines

A Porcine Aortic Endothelial Cell (PAEC) was kindly provided by S. Miyagawa. Obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) were pig cell lines PK15 (PTA-8244) and PT-K75 (CRL-2528); human cell lines HEK-293 (CRL-1573), U373 MG (HTB-17), Jurkat (TIB-152), HeLa (CCL-2), and Tera-1 (HTB-105); African green monkey kidney cell lines Vero (CCL-81), COS-1 (CRL-1650), and BGM (PTA-4594); mouse cell lines NIH/3T3 (CRL-1658) and J774A.1 (TIB67); mink cell line MiCl1 (CCL-64.1); and cat cell line PG-4 (CRL-2032).

1-2. Animals

Four miniature pig breeds were included in this study. Designated pathogen-free (DPF) SNU miniature pigs breed were bred in Centers for Animal Resource Development (CARD) in Seoul National University; two specific pathogen-free (SPF) breeds of PWG pig breed were supplied by PWG Genetics in Singapore; and Optipharm pig breed was provided by Optipharm Medipig in South Korea. Also,

KU miniature pigs which were of the same lineage as SNU pigs but grown in a different facility were obtained from Konkuk University. This study was approved according to the National Institute of Health guidelines and the Animal Care and Use Committee (IACUC: 12-0374-C2A2) of the Clinical Research Institute in Seoul National University Hospital AAALAC accredited facility.

1-3. Xenotransplanted Primate Blood and Tissues

Recipients of NHP were *Rhesus macaque* and their samples were obtained from Translational Xenotransplantation Research Center (TXRC). Peripheral blood leukocytes (PBL) of 5 each islet and cornea xenotransplanted primates were prepared by the use of RBC lysis protocol. Tissues from one each islet and cornea xenotransplanted primates were obtained after the xenotransplantation experiments were ended.

1-4. Isolation of Genomic DNA (gDNA)

Tissues and PBL cells from xenotransplanted primates were stored at -80°C and cell lines were stocked in liquid nitrogen until used. gDNA was extracted with the use of QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's manual.

2. Primers and Probes

The primers and probes (Bioneer, Seoul, Korea) for the detection of target genes are indicated in Table 1. Three kinds of labeled dyes such as Cyanine 5 (Cy5) – BHQ2 for PERV *pol*, 4-5-Dichloro carboxyfluorescein (JOE) – BHQ1 for pig CO Ⅱ, and fluorescein (FAM) –BHQ1 for monkey GAPDH were designed using primer Express 3.0.1 (Applied Biosystems, Foster City, CA, USA).

3. Cloning of Positive Control Gene

PERV *pol*, CO Ⅱ and GAPDH were cloned into T&A Cloning vector using T&A Cloning Kit (Real Biotech Corporation, Taipei, Taiwan). The target of PERV-*pol* sequence (GenBank: HM131062.1 and HM131068.1) was derived from sequences of the gDNA of SNU miniature pig PBL while CO Ⅱ (GenBank: AP003428.1) is derived from those of PK15 cell line. GAPDH gene (GenBank: AY624140.1 and KJ891221.1) originated from Cos-1 cell line and targeting the conserved region of human and monkey for the real-time PCR.

4. Multiplex Real-time PCR

TaqMan-based quantitative real-time PCR assays were performed to

detect multiple targets. TaqMan universal PCR master mix II (Applied Biosystems, Foster City, CA, USA) containing Uracil-N-Glycosylase (UNG) was used to degrade carry-over DNA which was a product from previous reactions. Ten pmoles of primers and probes per 20µl reaction were used to detect copy limit for three positive target sequences. The cycles of PCR reactions were at 50°C for 2m for UNG incubation, at 95°C for 10m for polymerase activation and 40 cycles of repetition at 95°C for 15m for denaturation, and at 60°C for 1m for annealing/extension stages.

5. Statistical Analysis

Statistical analysis was performed using the 7500 Software version 2.0.6 (Applied Biosystems, Foster City, CA, USA). A threshold of C_T was set on 0.02 for both singleplex real-time PCR and multiplex real-time PCR. C_T Mean and C_T standard deviation (SD) were computed by the software and coefficient of variation (CV; %) and calculated using the results of C_T Mean and C_T SD. Serial dilutions of each consensus clone, corresponding to 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, and 10 molecules per reaction were used to generate standard curves.

RESULTS

1. Specificity

Specificity of gene amplification by of real-time PCR was confirmed experimentally on the cell of 6 species. PERV *pol* for the PCR targeted the conserved region referenced on the sequences deposited in GenBank data (Figure 1). The results of *pol* and CO II were amplified positively in pig cell lines PK15, PAEC, and PK-K75 but not in human, monkey, mouse, mink, and cat cell lines. The amplification of human GAPDH real-time PCR was positive in human cell lines HEK-293, U373MG, Jurkat, HeLa, and Tera-1 and monkey cell lines Cos-1, Vero, and BGM whereas pig cell lines were not amplified (Figure 2).

2. Sensitivity

The sensitivity tests were examined ranging from 1 to 1×10^7 copies per reaction. The detection limits of *pol*, CO II, and GAPDH were 5 copies per 20μl reaction by singleplex real-time PCR, while they were 5, 5-10, and 5-10 copies, respectively by multiplex real-time PCR (Figure 3).

3. Intra-assay

Intra-assay precision was determined with the observed values within the same plate. From three experiments (n = 3) were measured the mean values and standard deviation (SD) based on threshold cycle (CT). The coefficient of variations (CV) (%) of *pol*, CO II and GAPDH were 0.2-3.0%, 0.3-2.4% and 0.4-5.3% by singleplex real-time PCR, respectively (Table 2-4). CVs of *pol*, CO II and GAPDH were 0.4-1.6%, 0.2-1.8% and 0.2-3.5% by multiplex real-time PCR, respectively (Tables 2-4). CVs of intra-assay reflected the precision of instruments and hands by the user and it is acceptable when the percentage of CVs is less than 10.0.

4. Inter-assay

Inter-assay reproducibility was evaluated triplicate in three independent experiments. The coefficient of variations (CV) (%) of *pol*, CO II and GAPDH were 0.9-5.0%, 0.5-8.3% and 1.2-3.8% by singleplex real-time PCR, respectively (Table 5-7). CVs of *pol*, CO II and GAPDH were 1.0-5.0%, 0.3-5.2% and 0.3-4.1% by multiplex real-time PCR, respectively. (Tables 5-7). Generally, below 15% CVs of inter-assay are acceptable to be determined. These results of inter-assays showed the repeatability of the repeated experiments on the same samples.

5. Linearity

Linear range of CT was determined using the results in serially 10-fold diluted samples of each positive control plasmid. The standard curves were created by the copy numbers in each sample on log scale quantity to CT values (Figure 4). All correlation coefficient of standard curves were 0.9966-0.9993 which fulfilled the criteria of $R^2 \geq 0.99$. The efficiency of all real-time PCR amplifications was from 92.1 to 99.1% which fulfilled 90–110% of amplification efficiency. These results demonstrated that the linearity of real-time PCR assays met the proposed analytical method.

6. Detection of PERV Proviral Level in Donor Pigs

Proviral level of PERV was determined by duplex real-time PCR of *pol* and CO II. SNU and KU miniature pigs which are from the same lineage represented that the ratio of *pol* to CO II was 5.8 and 4.9-fold higher than that of PK15, respectively. On the other hand, the ratio in PWG and Optipham pigs was approximately 3-fold higher than that of the reference. There was variation in individual pigs even in the same breed but the variation was the least in KU breed (Figure 5).

7. Monitoring of PERV Infectivity in Xenotransplated Primates

All PBL and tissue samples from primates with pig islets xenotransplanted were positive of GAPDH, which meant that all samples were of proper species for amplification. PERV *pol* was below the detection limit from all samples which indicated that productive PERV infection did not happen (Tables 8 and 9). Fourteen copies of CO II were detected in the liver of R041 which had a xenotransplantation of islets (Table 9).

Table 1. Characteristics of primers and probes for real-time PCR

Primer/probe	Sequence	Amplicon size (bp)	GenBank accession
pol real-time F	5`- CTG CAG CAG TTG GTC AGA ACA TC -3`	101	HM131062 HM131068
pol real-time R	5`- GCA TTA ACC AGC TGG CAG G -3'		
pol real-time probe	5`-Cy5- AGT GGC TGA CTC GGT GG -BHQ2-3'		
CO II real-time F	5`- CCC TTT CCA ACT AGG CTT CCA -3`	69	AP003428
CO II real-time R	5`- TGA TCG TGA AAG TGT AGG AGT TCT TC -3`		
CO II real-time probe	5`-JOE- ACG CCA CTT CAC CCA T -BHQ1 -3`		
GAPDH real-time F	5`- GCA TCC TGG GCT ACA CTG AG -3`	104	AY624140 KJ891221
GAPDH real-time R	5`- AAA GTG GTC GTT GAG GGC A -3`		
GAPDH real-time probe	5`-FAM- AGC GAC ACC CAC TCT TC -BHQ1-3`		

bp, base pairs; F, forward; R, reverse

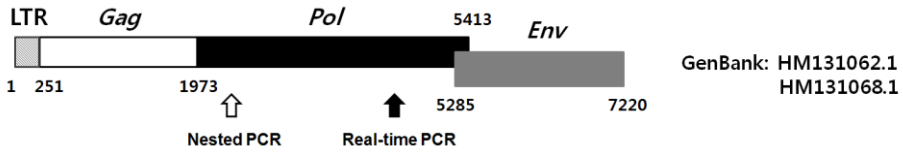


Figure 1. PERV *pol* constructs derived from SNU miniature pigs

Both nested PCR and real-time PCR include the conserved region of *pol* region referenced in GenBank data (HM131062.1 and HM131068.1). The sites of target region are noted for nested PCR (24) by white arrow and for the real-time PCR by black arrow. An inverted triangle illustrates the insertion sequences from other origins.

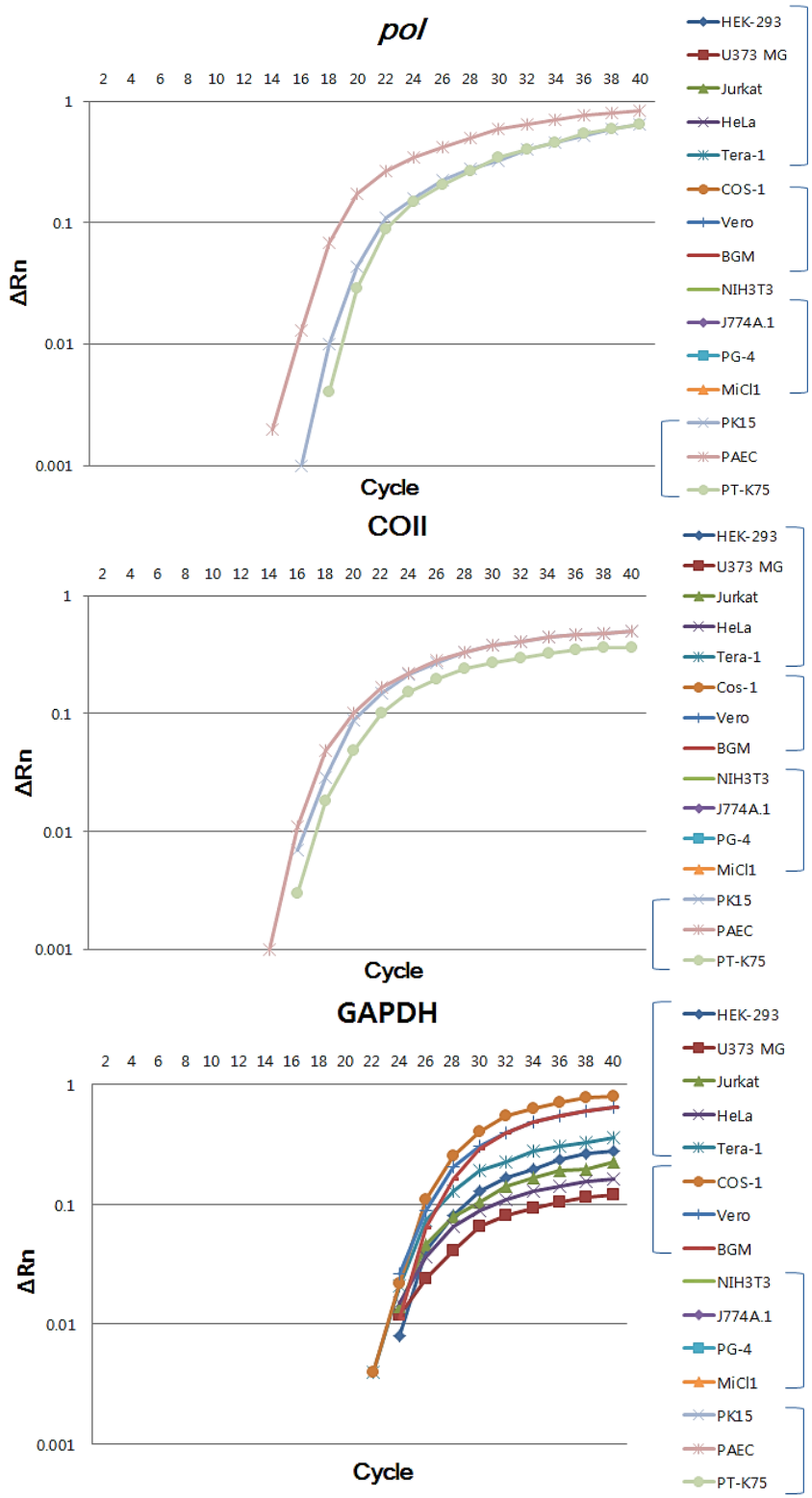

pol
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
1
0.1
0.01
0.001
ΔRn
Cycle
HEK-293
U373 MG
Jurkat
HeLa
Tera-1
COS-1
Vero
BGM
NIH3T3
J774A.1
PG-4
MiCl1
PK15
PAEC
PT-K75
COII
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
1
0.1
0.01
0.001
ΔRn
Cycle
HEK-293
U373 MG
Jurkat
HeLa
Tera-1
Cos-1
Vero
BGM
NIH3T3
J774A.1
PG-4
MiCl1
PK15
PAEC
PT-K75
GAPDH
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
1
0.1
0.01
0.001
ΔRn
Cycle
HEK-293
U373 MG
Jurkat
HeLa
Tera-1
COS-1
Vero
BGM
NIH3T3
J774A.1
PG-4
MiCl1
PK15
PAEC
PT-K75

Figure 2. Specific amplification of target genes originated from the different species in cell lines by real-time PCR

The DNA concentration of all samples was set at 50ng per reaction. The plots represent the amplification of PCR products following the cycles. PK15, PAEC, and PT-K75 cell lines (pig) showed amplified products in the reactions of *pol* and COⅡ but not in the reaction of GAPDH. HEK-293, U373 MG, Jurkat, HeLa and Tera-1 (human) and Cos-1, Vero, and BGM (African green monkey) cell lines showed amplified products in the reaction of GAPDH but not in the reactions of *pol* and COⅡ. The other cell lines such as NIH/3T3, J774A.1 (mouse), PG-4 (cat), and MiCl1 (mink) did not show any amplified products by real-time PCR (Rn, natural logarithm).

pol
COII
GAPDH
Singleplex
Multiplex

Figure 3. Detection limits by real-time PCR with the positive control plasmid

Detection limits of *pol* were 5 copies by both singleplex and multiplex real-time PCR. Detection limits of CO II and GAPDH were 5 and 5-10 copies by singleplex and multiplex real-time PCR, respectively. The threshold of cycle was set on 0.02 in exponential phase to compare singleplex with multiplex real-time PCR.

Table 2. Intra-assay of *pol* by real-time PCR.

pol singleplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	14.0	13.6	13.8	13.8	0.2	1.4
10^6	17.0	17.5	17.3	17.3	0.2	1.4
10^5	20.9	21.0	21.0	21.0	0.1	0.4
10^4	24.6	24.6	24.6	24.6	0.0	0.2
10^3	28.4	28.5	28.8	28.5	0.2	0.7
10^2	31.9	32.2	32.4	32.1	0.3	0.8
10	36.2	38.2	36.2	36.9	1.1	3.0
5	38.3	37.0	36.7	37.3	0.9	2.4

pol multiplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	14.0	13.9	13.9	13.9	0.1	0.5
10^6	17.5	17.1	17.7	17.4	0.3	1.6
10^5	21.0	20.7	20.9	20.9	0.1	0.5
10^4	24.6	24.8	24.8	24.7	0.1	0.5
10^3	28.3	27.9	27.9	28.0	0.2	0.8
10^2	30.8	30.5	30.2	30.5	0.3	0.9
10	33.2	33.0	33.0	33.1	0.1	0.4
5	33.8	33.7	33.9	33.8	0.1	0.4

C_T, threshold cycle; SD, standard deviation; CV, coefficient of variation

Table 3. Intra-assay of COⅡ by real-time PCR.

COⅡ singleplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	15.6	15.7	15.9	15.7	0.2	1.0
10^6	18.9	18.7	18.9	18.8	0.1	0.5
10^5	22.5	22.5	22.4	22.5	0.1	0.3
10^4	25.6	25.8	25.9	25.8	0.2	0.6
10^3	29.3	29.6	29.8	29.6	0.2	0.8
10^2	33.0	33.2	33.4	33.2	0.2	0.6
10	36.3	36.6	37.4	36.7	0.6	1.5
5	36.3	38.0	37.8	37.4	0.9	2.4

COⅡ multiplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	15.0	15.3	15.3	15.2	0.2	1.2
10^6	18.5	18.1	18.4	18.3	0.2	1.0
10^5	21.7	21.4	21.5	21.5	0.2	0.9
10^4	24.9	24.9	24.8	24.9	0.1	0.2
10^3	29.1	29.0	28.9	29.0	0.1	0.3
10^2	31.4	31.4	30.9	31.3	0.3	0.9
10	34.4	34.1	34.3	34.3	0.2	0.4
5	35.9	35.3	36.6	36.0	0.6	1.8

C_T, threshold cycle; SD, standard deviation; CV, coefficient of variation

Table 4. Intra-assay of GAPDH by real-time PCR.

GAPDH singleplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	13.1	13.7	13.5	13.4	0.3	2.5
10^6	17.9	17.6	17.8	17.7	0.2	0.9
10^5	19.9	20.3	20.4	20.2	0.3	1.5
10^4	23.8	24.5	24.4	24.2	0.3	1.4
10^3	28.4	28.6	28.4	28.5	0.1	0.4
10^2	31.4	31.7	31.6	31.6	0.2	0.5
10	36.3	32.7	34.6	34.6	1.8	5.3
5	37.8	35.4	35.3	36.1	1.4	3.9

GAPDH multiplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	13.4	13.5	13.1	13.3	0.2	1.5
10^6	16.8	16.7	16.8	16.8	0.0	0.2
10^5	20.7	20.6	20.2	20.5	0.3	1.4
10^4	23.8	23.7	23.6	23.7	0.1	0.3
10^3	27.8	27.5	27.3	27.6	0.2	0.8
10^2	30.2	30.7	30.2	30.3	0.3	1.0
10	35.2	33.6	32.8	33.9	1.2	3.5
5	34.1	34.6	34.0	34.2	0.3	0.9

C_T, threshold cycle; SD, standard deviation; CV, coefficient of variation

Table 5. Inter-assay of *pol* by real-time PCR.

pol singleplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	14.0	13.8	13.6	13.8	0.2	1.5
10^6	17.0	17.3	17.1	17.1	0.2	0.9
10^5	20.4	21.0	20.5	20.6	0.3	1.5
10^4	23.5	24.6	24.3	24.1	0.6	2.4
10^3	27.3	28.5	27.7	27.9	0.6	2.2
10^2	31.4	32.1	31.2	31.6	0.5	1.6
10	33.4	36.9	35.8	35.3	1.8	5.0
5	36.4	37.3	36.2	36.6	0.6	1.7

pol multiplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	14.5	13.3	13.9	13.9	0.6	4.6
10^6	17.4	17.1	17.4	17.3	0.2	1.1
10^5	22.4	20.6	20.9	21.3	1.0	4.5
10^4	25.6	25.6	24.7	25.3	0.5	1.9
10^3	28.6	28.2	28.0	28.3	0.3	1.0
10^2	32.2	32.6	30.5	31.8	1.1	3.5
10	35.9	35.2	33.1	34.7	1.5	4.3
5	37.0	36.8	33.8	35.9	1.8	5.0

C_T, threshold cycle; SD, standard deviation; CV, coefficient of variation

Table 6. Inter-assay of COⅡ by real-time PCR.

COⅡ singleplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	14.9	15.7	16.7	15.7	0.9	5.7
10^6	18.0	18.8	21.1	19.3	1.6	8.3
10^5	21.8	22.5	23.9	22.7	1.1	4.6
10^4	25.1	25.8	27.2	26.0	1.1	4.1
10^3	28.4	29.6	30.3	29.4	1.0	3.2
10^2	32.3	33.2	34.6	33.4	1.2	3.5
10	33.6	36.7	36.0	35.5	1.7	4.7
5	37.7	37.4	37.3	37.5	0.2	0.5

COⅡ multiplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	14.3	14.7	15.2	14.7	0.4	2.5
10^6	17.5	18.4	18.3	18.1	0.4	2.1
10^5	21.4	21.6	21.5	21.5	0.1	0.4
10^4	24.8	24.7	24.9	24.8	0.1	0.3
10^3	28.0	29.0	29.0	28.7	0.5	1.6
10^2	31.5	33.1	31.3	32.0	0.8	2.5
10	35.0	38.5	34.3	35.9	1.9	5.2
5	-	-	36.0	36.0	-	-

C_T, threshold cycle; SD, standard deviation; CV, coefficient of variation

Table 7. Inter-assay of GAPDH by real-time PCR.

GAPDH singleplex

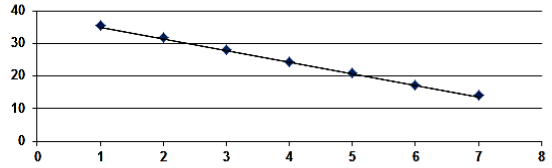
Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	13.4	14.5	14.1	14.0	0.5	3.8
10^6	17.7	18.1	17.0	17.6	0.5	3.0
10^5	20.2	21.6	20.5	20.8	0.7	3.5
10^4	24.2	24.8	24.3	24.4	0.3	1.2
10^3	28.5	29.0	28.3	28.6	0.4	1.3
10^2	31.6	33.5	30.6	31.9	1.5	4.6
10	34.6	36.6	34.5	35.2	1.2	3.3
5	36.1	36.8	34.8	35.9	1.0	2.9

GAPDH multiplex

Copy No.	C_T 1	C_T 2	C_T 3	C_T Mean	C_T SD	C_T CV
10^7	13.3	13.6	13.6	13.5	0.2	1.2
10^6	16.8	17.3	16.7	16.9	0.4	2.1
10^5	20.5	20.7	20.0	20.4	0.3	1.6
10^4	23.7	23.6	23.5	23.6	0.1	0.4
10^3	27.6	28.0	28.8	28.1	0.6	2.3
10^2	30.3	31.8	30.2	30.8	0.9	2.9
10	33.9	35.8	33.1	34.3	1.4	4.1
5	34.2	-	34.1	34.2	0.1	0.3

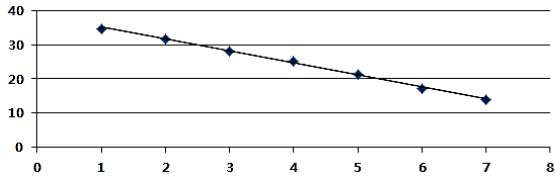
C_T, threshold cycle; SD, standard deviation; CV, coefficient of variation

pol singleplex



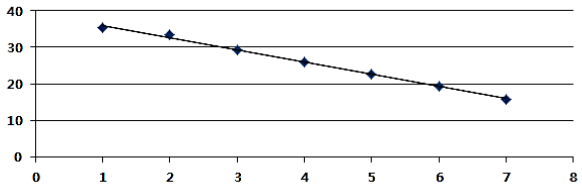
Correlation Coefficient: 0.9996
Slope: -3.6038
Intercept: 38.754
y = -3.6038x + 38.754
R^2 = 0.9996
PCR Efficiency: 92.1%

pol multiplex



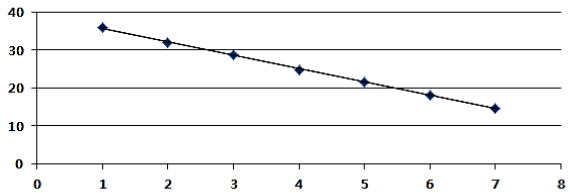
Correlation Coefficient: 0.9976
Slope: -3.5128
Intercept: 38.696
y = -3.5128x + 38.696
R^2 = 0.9976
PCR Efficiency: 93.8%

COⅡ singleplex



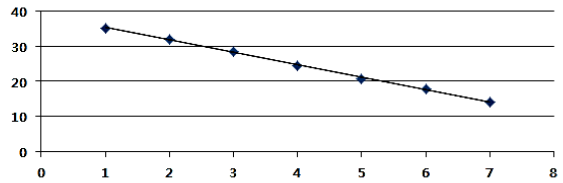
Correlation Coefficient: 0.9973
Slope: -3.3534
Intercept: 39.42
y = -3.3534x + 39.42
R^2 = 0.9973
PCR Efficiency: 99.1%

COⅡ multiplex



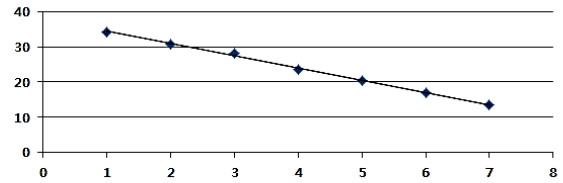
Correlation Coefficient: 0.9993
Slope: -3.5158
Intercept: 39.167
y = -3.5185x + 39.164
R^2 = 0.9993
PCR Efficiency: 94.4%

GAPDH singleplex



Correlation Coefficient: 0.9987
Slope: -3.5477
Intercept: 38.874
y = -3.5477x + 38.874
R^2 = 0.9987
PCR Efficiency: 93.8%

GAPDH multiplex



Correlation Coefficient: 0.9982
Slope: -3.4877
Intercept: 37.891
y = -3.5185x + 39.164
R^2 = 0.9982
PCR Efficiency: 95.2%

Threshold Cycle

Log Starting Quantity, copy number

Figure 4. Standard curves for the detection of target genes by the singleplex and multiplex real-time PCR

Linearity of standard curves shows the logarithmic quantity of target genes to threshold cycle (C_T). All correlation coefficients were higher than 0.99 of reliability, and slopes were lower than -3.32, the predicted value of natural log scale. All the results of PCR efficiency were higher than 92% which met the criteria of the expectation (90-110%).

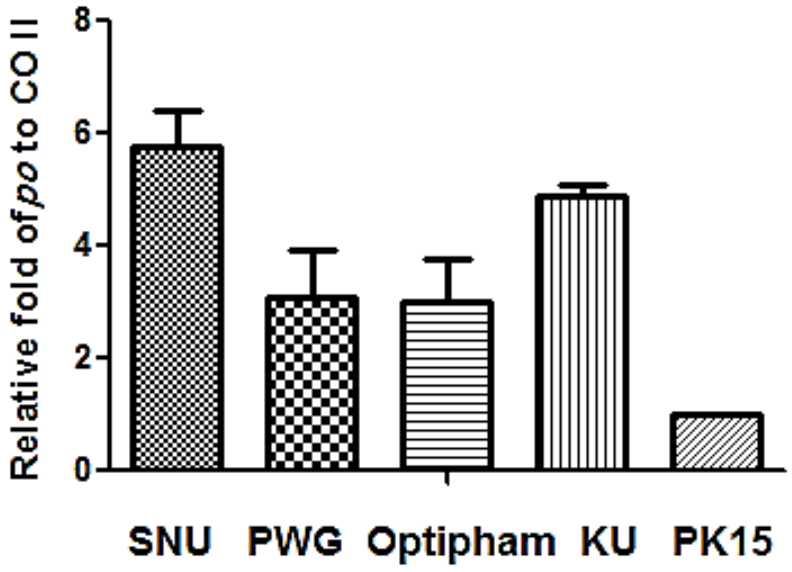


Figure 5. Proviral level of PERV *pol* to porcine CO II in various pig breeds compared to PK15

Bar graphs using CT values show the relative ratio of *pol* to CO II when the ratio of PK15, 1.0, was used as the standard. Bars indicate the mean of each breed, and error bars represent the SD derived from the mean. Three individual pigs in each breed were analyzed by *pol* and CO II duplex real-time PCR (SD, standard deviation).

Table 8. Results of PERV infectivity testing in PBL of xenografted non-human primated (NHP) by multiplex real-time PCR.

Time after xenotransplantation	Copy Numbers											
	Pre-operation			3M			6M			12M		
Monkey No./ Targets	GAPDH	*pol*	CO II	GAPDH	*pol*	CO II	GAPDH	*pol*	CO II	GAPDH	*pol*	CO II
PBL of islet-xenotransplanted NHP												
R051	1096	-	-	1292	-	-	252	-	-	277	-	-
R080	2421	-	-	1818	-	-	43	-	-	409	-	-
R082	2624	-	-	436	-	-	979	-	-	NT	NT	NT
R084	802	-	-	389	-	-	10245	-	-	1691	-	-
R089	1277	-	-	878	-	-	1343	-	-	NT	NT	NT
PBL of cornea-xenotransplanted NHP												
R016	127	-	-	1035 (2M)	-	-	NT	NT	NT	NT	NT	NT
R019	68	-	-	4248	-	-	138	-	-	NT	NT	NT
R023	2612	-	-	1177	-	-	424	-	-	NT	NT	NT
R062	NT	NT	NT	1209	-	-	1532	-	-	2474	-	-
R065	NT	NT	NT	880	-	-	11945	-	-	6910	-	-

-, below detection limit; NT, not tested due to no sample.

Table 9. Results of PERV infectivity testing in tissues of xenografted non-human primates (NHP) by multiplex real-time PCR.

Targets/ tissues	Copy Numbers													
	Brain	Liver	Lymph node	Heart	Large intestine	Small intestine	Spleen	Lung	Pancreas	Stomach	Muscle	Skin	Kidney	Urinary bladder
Islet xenotransplanted NHP: R041														
GAPDH	1006	1799	877	1190	1285	816	2240	802	594	NT	NT	NT	NT	NT
pol	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT
Co II	-	14	-	-	-	-	-	-	-	NT	NT	NT	NT	NT
Cornea xenotransplanted NHP: R032														
GAPDH	541	3891	898	492	3918	4107	2577	1706	NT	164	1927	2133	3839	567
pol	-	-	-	-	-	-	-	-	NT	-	-	-	-	-
Co II	-	-	-	-	-	-	-	-	NT	-	-	-	-	-

-, below detection limit; NT, not tested due to no sample.

DISCUSSION

In this study, the sensitive and specific detection of PERV was established in various types of cells and tissues by multiplex real-time PCR. The detection of PERV is critical in pre-clinical and clinical trials in reference to the consensus statement for the xenotransplantation (33, 38).

The specificity of each target was determined by the detection of PERV in cells from six species. The detection of monkey GAPDH was confined to monkey cell lines and human cell lines even though amplification plots differed in cells of each species. Previous other work published with a comparison of primate and human gene expression also showed that there were no significant differences in GAPDH (39). PERV *pol* and pig COII were specific to pig cell lines. Pig COII was important in detecting sample contamination from pig originated cells (24).

The results of C_T values were slightly affected by multiplex real-time PCR compared to singleplex real-time PCR. Nevertheless, one tube reaction by multiplex real-time PCR had advantages in the reduction of error rate compared to using separate tubes.

The precision test of intra-, inter-, and linearity assays demonstrated

that this multiplex real-time PCR analysis was reliable to apply to PERV infectivity. All of the results were followed by the criteria of the expectations (40, 41). Therefore, these data demonstrated that the setting of multiplex PCR analysis was qualified to determine the PERV infectivity test of pre-clinical and clinical trials.

PERV proviral level tests on donor pigs revealed the PERV load. There were variations from breed to breed and individual to individual within the same breed. Other data quantifying PERV ratio using quantitative real-time PCR indicated that there were differences in breeds and even organs in the same individual (37, 42-44). Comparison of *pol* in diverse pig breeds demonstrated that SNU miniature pigs seemed to have a high level of PERV presence. The selection of the conserved target site was based on GenBank data of PERV constructs originating from SNU miniature pigs (Figure 1). Defective constructs of PERV were found in many pig strains (44, 45) so the target site might not be detected in other strains.

PERV infectivity test using multiplex real-time PCR showed that all xenotransplated primates were not infected by PERV derived from pig. These results supported previous data that there was no evidence of PERV infectivity in other hosts (25, 27). However, the liver sample from the islet transplanted primate detected 14 copies of CO II. In this case, microchimerism or donor mitochondria transfer to recipient might have occurred because islet cells were inoculated in the liver for

transplantation (31). If the result was detected in the PERV *pol* as well, it would be microchimerism or mix of cells between different organisms (28, 29). That is why examination of pig mitochondrial gene must be accompanied with PERV infection test. Also, the standard procedures from sample to multiplex real-time PCR reaction were required to be a diagnosis tool. In sight of results of monkey GAPDH used as the internal control, some samples were detected in the low copy numbers. It might be the insufficient concentration on the performance of PERV infectivity test. Therefore, standard cut-off of GAPDH copy numbers needs to be determined for the setting.

In conclusion, multiplex real-time PCR was an effective discrimination tool in both the screening of pig strains and monitoring of follow-up from xenograft model in pre-clinical test. This study can be useful for monitoring biosafety in xenotransplantation.

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국문 초록

돼지 내인성 레트로바이러스 (Porcine Endogenous Retrovirus; PERV)는 이종장기이식에 있어서 극복해야 할 과제이다. 그러므로 이식 전 검사로 적절한 공여 동물을 선택하는 것과 이식 후 수여자에서 바이러스의 레벨을 확인하는 것은 안전성 측면에서 중요하다. 이러한 확인 방법으로 바이러스의 감염 확인을 위한 *pol* 유전자, 돼지세포 오염 구별을 위한 돼지 사립체 사이토크롬 산화효소 (mitochondrial cytochrome oxidase Ⅱ; COⅡ) 유전자 그리고 내부대조로써 원숭이의 글리세르알데히드-3-인산 디히드로게나아제 (gyceraldehyde-3-phosphate dehydrogenase; GAPDH) 유전자를 표적으로 하는 다중 실시간 중합효소 연쇄반응 (multiplex real-time PCR)을 개발하였다. PERV에 사용되는 프라이머와 프로브는 기존 연구에서 밝혀진 SNU 미니돼지의 유전자 내 공통으로 보존된 부위를 선택하였을지라도, 다른 돼지 종과 여러 암 세포 주에서도 검출할 수 있는 것을 확인하였다. 게다가, 검출한계는 20μl의 반응 당 유전자 5-10개 이었고, 기존에 사용되고 있던 방법인 네스티드 중합효소 연쇄반응 (nested PCR)과 비교하여 비슷한 정도인 것을 확인하였다. 다중검출이 단일 검출보다 민감성이 다소 떨어지더라도 한 번에 반응을 확인하여 오류를 낮출 수 있는 장점이 있다. 다중 실시간 중합효소 연쇄반응을 여러 돼지 종에서 적용하여 COⅡ와 비교해 돼지 PERV *pol*의 비율

을 구한 결과, PK15 암 세포주와 비교하여 SNU 미니돼지에서 평균 5.8배 높은 것을 확인하였다. 또한, PERV의 감염여부를 이종 이식된 원숭이 혈액과 조직에서 검사한 결과, 모든 샘플에서 검출되지 않았음을 확인하였다. 이러한 결과들은 세 가지 형광의 표지 된 실시간 중합효소 연쇄반응이 공여 돼지 내에서 PERV의 레벨을 확인하여 적절한 공여개체를 선택하게 할 수 있을 뿐만 아니라, 이종이식 후, 영장류 전임상 실험과 사람 환자에게 적용하여 바이러스가 수여자에게 전이될 수 있는지 확인할 수 있는 두 가지의 역할을 할 수 있음을 보여준다.

주 요 어: 이종장기이식, 돼지 내인성 레트로바이러스, 다중 실시간 중합효소 연쇄반응, 프로바이러스 양, 감염성 검사

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